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Allozyme discrimination of three species of *Loricariichthys* (Siluriformes: Loricariidae) from Southern Brazil[1]

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Allozyme discrimination of three species of *Loricariichthys* (Siluriformes: Loricariidae) from Southern Brazil. - Three species of armored catfish of the genus *Loricariichthys* were clearly discriminated by starch gel isozyme electrophoresis on heart, liver and muscle tissues. We scored 21 enzyme loci from 14 isozyme systems (ACP, ADH, AAT, EST, GCDH, G3PDH, G6PDH, GPI, ICDH, LDH, MDH, MDHP, PGM and SOD). Expected mean heterozygosity (H_e) ranged from 0.023 in *Loricariichthys anus* to 0.050 in *L. platymetopon* and 0.035 in *Loricariichthys* sp. The percentage of polymorphic loci ($P_{0.99}$) was 14.3% for the three analyzed species. Nei's genetic identity (I) was found to be 0.663 between *Loricariichthys anus* and *Loricariichthys* sp., 0.329 between *L. anus* and *L. platymetopon*, and 0.478 between *L. platymetopon* and *Loricariichthys* sp. Genetic variability, tissue specific differences, as well as applicability of isozyme electrophoresis as a tool for neotropical fish systematists are discussed.

Key-words: Genetic diversity - isozymes - polymorphism - Siluriformes - taxonomy - systematics - fish - *Loricariichthys*.

INTRODUCTION

The freshwater fish fauna of South America is the richest of all the continents and rivals in diversity with the coral reef fishes. In a recent estimate of the diversity of neotropical ichthyofauna Schaefer (1998) suggested that the total number of species of fish in this region may reach about eight thousands. The gaps in the systematic knowledge of neotropical fishes are as great as the number of species, and can be sustained principally by the lack of overall representation in scientific collections. One of the many difficulties from dealing with neotropical fishes is related to the great

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number of forms in each taxonomic group and to the great similarity found among species of the same genus. The family Loricariidae is the largest among the Siluriformes, with many species difficult to identify in several genera. In *Loricariichthys* Bleeker, 1862 as is usually the case for fish systematics and taxonomy, morphological characters forms the bulk of data used in descriptions as well as in species diagnoses (Reis & Pereira, in press). The genus *Loricariichthys* comprises 16 valid species (Isbrücker, 1980) and do not diverge from other neotropical taxa of fishes in regard to the gaps in the systematic knowledge. Thus, we search auxiliary tools for the morphological criteria. Different cytogenetic, biochemical and molecular techniques have been developed and used to assist in the diagnosis and species identification. As suggested by Thorpe & Solé-Cava (1994), isozyme electrophoresis can become an extremely useful tool when combined with morphological methods for the discrimination of closely related species. In this work we examine the level of genetic identity and variability among three species of *Loricariichthys* through allozyme electrophoresis, assaying loci and tissue expression. These are first and partial results from an attempt of the first author to form a bulk of allozyme data of some Loricariidae catfishes from southern Brazil, with the objective of finding genetic markers that could help to resolve questions on the systematics of this family. We also aim to corroborate the use of allozyme electrophoresis as a tool for the discrimination of morphologically similar species of Loricariidae.

MATERIALS AND METHODS

After withdraw the tissues, the specimens were fixed in 10% formalin and they are alcohol preserved in the synoptic collection of Nupélia at the State University of Maringá, Brazil. Voucher specimens are deposited at the Museu de Zoologia da PUC, Porto Alegre, Brazil, *L. platymetopon* MCP25476 and MCP25749; *Loricariichthys* sp. MCP25747 and MCP25748, and also at the Muséum d'Histoire Naturelle, Geneva (Switzerland). A list of specimens was arranged in the text as follow: locality, town, collecting date, number of specimens (if there are more than one) between parenthesis and the standard length in centimeters (the smallest and biggest if there are more than one in the lot).

List of specimens studied

Brazil, Paraná State:

Loricariichthys platymetopon: Vila Ipiranga (25°14'12"S/54°14'02"W), rio Ocoí, rio Paraná basin, São Miguel do Iguaçu, 10.ix.1998, 27.8; 16-17.ix.1998, (4) 22.7-26.4; Passo Côe (25°21'64"S/54°24'50"W), Itaipu lake reservoir, Foz do Iguaçu, 15.ix.1998, (2) 23.7-26.3; São Vicente (25°01'20"S/54°23'57"W), Itaipu lake reservoir (25°01'20'S/54°23'57''W), rio Paraná basin, Santa Helena, 13.ix.1998, 30.1; Ponto do Chico Barbudo (25°22'32"S/54°26'05"W) Itaipu lake reservoir, rio Paraná basin, Santa Terezinha do Itaipu, 14.ix.1998, (4) 22.9-27.8; Esquina Gaucha (25°17'42"S/54°19'20"W), Itaipu lake reservoir, rio Paraná basin, Itaipulândia, 15.ix.1998, 28.9; Itaipu lake reservoir (24°28'19"S/54°18'49"W), rio Paraná basin, Marechal Cândido Rondom, 18.ix.1998, (3) 24.6-31.6.

Loricariichthys sp.: Vila Ipiranga (25°14'12"S/54°14'02"W), rio Ocoí, rio Paraná basin, São Miguel do Iguaçu, 10.ix.1998, (5) 25.3-28.2; 17.ix.1998, (3) 223.7-25.4 Rio São Francisco Verdadeiro (24°40'32"S/54°13'92"W), rio Paraná basin, Pato Bragado, 11.ix.1998, (2) 19.5-25.3;

Ponto do Chico Barbudo (25°22'32"S/54°26'05"W), Itaipu lake reservoir, rio Paraná basin, Santa Terezinha do Itaipu, 14.ix.1998, (2) 22.7-26.3; Esquina Gaucha (25°17'42"S/54°19'20"W), Itaipu lake reservoir, rio Paraná basin, Itaipulândia, 15.ix.1998, 27.9 .

Brazil, Rio Grande do Sul State:

Loricariichthys anus: Fortaleza lagoon (30°09'02"S/50°14'30"W), Cidreira, 20.iii.1999, (8) 25.7-26.4.

Samples of white skeletal muscle, liver and heart tissues were collected. The tissues were stored in liquid nitrogen until analysis. Samples of tissues were homogenized with plastic sticks, in microcentrifuge tubes with Tris/HCl 0.02 M, pH 7.5 buffer in 1:1 concentration.

When homogenizing liver samples, 0.5 ml of CCl_4 was added to precipitate the great amount of fat present in such tissue (Pasteur *et al.*, 1988). The homogenized samples were centrifugated at 25,000 rpm (44,720 x g) for 30 min. at temperatures between 1° C and 5° C. The supernatant fractions were applied to starch gel using straps of Whatman paper 3 MM®. Gels were prepared with corn starch (Penetrose®) 13% in a buffer solution. Two buffer systems were used for the electrophoresis: Tris-citrate, pH 7.0 (Shaw & Prasad, 1970) and Tris-borate-EDTA pH 8.3 (Boyer *et al.*, 1963). The electrophoresis was run during six hours at 5° C with 250 V for Tris-citrate gel and 450 V for Tris-borate-EDTA gel. Gels were then cut horizontally and each slice was incubated with a solution of appropriate staining for each enzyme. The staining procedures were those described by Aebersold *et al.* (1987), with the exception of the AAT which followed the procedures of Morizot & Schmidt (1990). The nomenclature used was that proposed by the International Union of Biochemistry and Molecular Biology (1992). The data were analyzed using Biosys 1 software (Swofford & Selander, 1981). The genetic interpretation of the enzymatic patterns was based on the quaternary structure of enzymes described by Ward *et al.* (1992).

RESULTS

Fourteen enzymatic systems of eight *Loricariichthys anus*, 16 *L. platymetopon* and 13 *Loricariichthys* sp. were analyzed, allowing the detection of 21 loci (Table 1). Electrophoretic patterns of enzymes in each tissue and their genetic interpretation for the three species analyzed are shown in Figure 1. Only the most common alleles for each species are represented.

The enzymes ACP, ADH, GCDH and G6PDH present a single band only in liver samples. Esterases are more active in the liver, but they are also expressed in other tissues, though less intensely. AAT, PGM and SOD zymograms show that these enzymes have the same activity in the three tissues and exhibit only one band.

The enzyme G3PDH presents three bands in white muscle, probably due to presence of two loci and the formation of a heterodimer interloci. Locus G3pdh-A also presents strong activity in heart and liver, while locus G3pdh-B has a weak expression in liver and is practically absent in heart tissues of the three species.

The enzyme GPI presents three regions of activity in the three species, as a result of expression of two loci Gpi-A and Gpi-B, and the intermediate region as corresponding to the formation of heterodimer interloci. In heart tissues the two loci

TABLE 1. Names, Number of Enzyme Commission (E.C.n°), tissues, buffers, interpreted quaternary structure (Q.S) and number of loci expressed in *Loricariichthys anus*, *L. platymetopon* and *Loricariichthys* sp. L = liver; M = muscle; H = Heart; TBE = Tris/borate/EDTA; TC = Tris/citrate.

Enzyme (Abbreviation)	E.C. n°	Tissue	Buffer	Q. S.	Loci
Acid phosphatase (ACP)	3.1.3.2	L	TC	Dimeric	1
Alcohol dehydrogenase (ADH)	1.1.1.1	L	TBE	Dimeric	1
Aspartate aminotransferase (AAT)	2.6.1.1	L, H, M	TBE	Dimeric	1
Esterase (EST)	3.1.1.1	L, H, M	TBE	Monomeric	1
Glucose 1-dehydrogenase - NAD+ (GCDH)	1.1.1.118	L	TBE	Dimeric	1
Glycerol-3-phosphate dehydrogenase (G3PDH)	1.1.1.8	L, H, M	TC	Dimeric	2
Glucose-6-phosphate dehydrogenase (G6PDH)	1.1.1.49	L	TBE	Tetrameric	1
Glucose-6-phosphate isomerase (GPI)	5.3.1.9	L, H, M	TC	Dimeric	2
Isocitrate dehydrogenase - NADP+ (ICDH)	1.1.1.42	L, H, M	TC	Dimeric	2
L-Lactate dehydrogenase (LDH)	1.1.1.27	H, M	TC	Tetrameric	2
Malate dehydrogenase (MDH)	1.1.1.37	L, H, M	TC	Dimeric	3
Malate dehydrogenase - NADP+ (MDHP)	1.1.1.40	L, H, M	TC	Tetrameric	2
Phosphoglucomutase (PGM)	5.4.2.2	L, H, M	TC	Monomeric	1
Superoxide dismutase (SOD)	1.15.1.1	L, H, M	TBE	Dimeric	1

present an equally intense activity. In liver, Gpi-A expresses itself with more intensity than that of Gpi-B, while the reverse is true for muscle tissues.

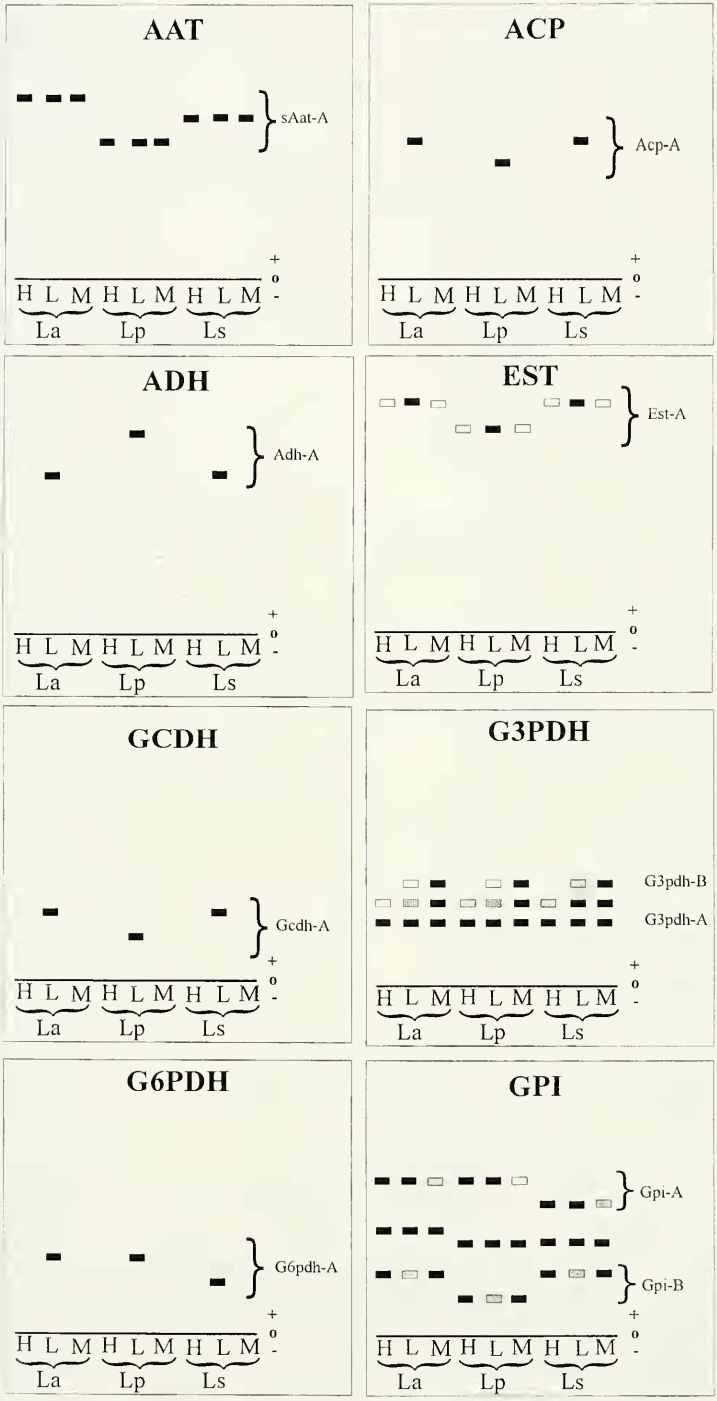
The tetrameric enzyme LDH presented five bands in muscle tissues, due to the expression of two loci, Ldh-A and Ldh-B, and three interloci heterotetramers. In heart only one band occurs, apparently corresponding to the A_1B_3 heterotetramer. A weak expression of heterotetramers and the absence of homotetramers in the three species studied were observed in liver tissues. In *L. anus* three weak bands (A_3B_1, A_2B_2 and A_1B_3), were observed in liver, while in *L. platymetopon* and *Loricariichthys* sp. a two banded pattern (A_2B_2 and A_1B_3) was observed.

The dimeric enzyme ICDH presented two loci with different tissue-expression for the three species. Locus mIcdh-A expressed itself in heart and muscle and was absent in liver tissues. Locus sIcdh-A presents bands in liver tissues only.

The dimeric enzyme MDH presents three loci for *Loricariichthys*. Locus sMdh-A has a greater intensity of expression in heart, and weaker intensity in liver and muscle tissues. mMdh-A locus expresses itself only in liver of *L. anus* and *Loricariichthys* sp. In *L. platymetopon*, despite a better resolution of the bands in liver, weak bands are also observed in heart and muscle tissues. sMdh-B locus expresses itself with greater intensity in heart and muscle tissues than in the liver.

Table 2 shows allelic frequencies of the three analyzed species. *Loricariichthys anus* is polymorphic at loci Adh-A, G3pdh-A, and Gpi-B; *L. platymetopon* is polymorphic at Gpi-A, Gpi-B, and sMdh-A, and *Loricariichthys* sp. is polymorphic at Gcdh-A, Gpi-A, and Pgm-A. Of the enzymes studied, eleven loci were diagnostic for

FIG. 1a. Schematic representation of enzymatic systems AAT, ACP, ADH, EST, GCDH, G3PDH, G6PDH and GPI of *L. anus* (La), *L. platymetopon* (Lp) and *Loricariichthys* sp. (Ls), and their genetic interpretation.


AAT
sAat-A
ACP
Acp-A
ADH
Adh-A
EST
Est-A
GCDH
Gcdh-A
G3PDH
G3pdh-B
G3pdh-A
G6PDH
G6pdh-A
GPI
Gpi-A
Gpi-B
H L M H L M H L M
La Lp Ls
+
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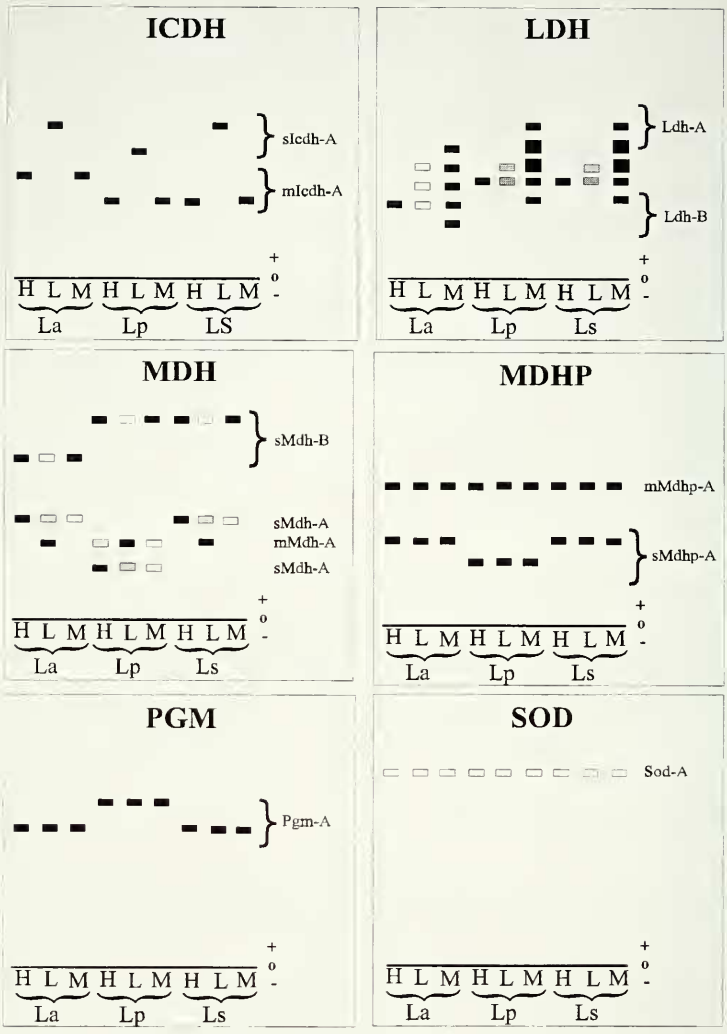

FIG. 1b. Schematic representation of enzymatic systems ICDH, LDH, MDH, MDHP, PGM and SOD of *L. anus* (La), *L. platymetopon* (Lp) and *Loricariichthys* sp. (Ls), and their genetic interpretation.

one or another of the three species. Loci sAat-A, Acp-A, Est-A, sIcdh-A, sMdh-A, and sMdhp-A are diagnostic for *L. platymetopon*, that is, they are fixed for different alleles. Loci sAat-A, mIcdh-A, Ldh-A, Ldh-B, and sMdh-B are diagnostic for *L. anus* and the loci sAat-A and G6pdh-A are diagnostic for *Loricariichthys* sp.

The genetic variability for the three species of *Loricariichthys* is presented in Table 3. All the polymorphic loci analyzed were in Hardy-Weinberg equilibrium. Unbiased genetic identity (I) and genetic distance (D) of Nei (1978) among the three species is represented in Table 4.

TABLE 2. Allelic frequencies observed for 21 loci in *L. anus*, *L. platymetopon* and *Loricariichthys* sp.

LOCUS	ALLELE	*L. anus* (n = 8)	*L. platymetopon* (n = 16)	*Loricariichthys* sp. (n = 13)
sAat-A	*a*	----	1,000	----
	b	----	----	1,000
	c	1,000	----	----
Acp-A	*a*	----	1,000	----
	b	1,000	----	1,000
Adh-A	*a*	0,875	----	1,000
	b	0,125	1,000	----
Est-A	*a*	----	1,000	----
	b	1,000	----	1,000
Gcdh-A	*a*	----	1,000	0,308
	b	1,000	----	0,692
G3pdh-A	*a*	0,063	----	----
	b	0,938	1,000	1,000
G3pdh-B	*a*	1,000	1,000	1,000
G6pdh-A	*a*	----	----	1,000
	b	1,000	1,000	----
Gpi-B	*a*	----	0,906	----
	b	0,938	0,094	1,000
	c	0,063	----	----
Gpi-A	*a*	----	0,125	0,885
	b	1,000	0,500	0,115
	c	----	0,375	----
mIcdh-A	*a*	----	1,000	1,000
	b	1,000	----	----
sIcdh-A	*a*	----	1,000	----
	b	1,000	----	1,000
Ldh-B	*a*	1,000	----	----
	b	----	1,000	1,000
Ldh-A	*a*	1,000	----	----
	b	----	1,000	1,000
sMdh-A	*a*	----	0,156	----
	b	----	0,844	----
	c	1,000	----	1,000
mMdh-A	*a*	1,000	1,000	1,000
sMdh-B	*a*	1,000	----	----
	b	----	1,000	1,000
sMdhp-A	*a*	----	1,000	----
	b	1,000	----	1,000
mMdhp-B	*a*	1,000	1,000	1,000
Pgm-A	*a*	1,000	----	0,962
	b	----	1,000	0,038
Sod-A	*a*	1,000	1,000	1,000

DISCUSSION

The usefulness of this technique as a tool for discrimination of similar species is confirmed, especially for fish systematists, whose morphological criteria are, not always, fully efficient to discriminate taxa. Isozyme electrophoresis constitutes a practical tool, given the easiness of its application, relative little need of specialized equipment, reduced cost, and the ability of obtaining data in a relatively short time (Buth & Murphy, 1999).

TABLE 3. Genetic variability measures for 21 loci of three *Loricariichthys* species. Number in parentheses are standard deviation. N = number of analyzed specimens; K = mean number of alleles per locus; $P_{0.99}$ = frequency of polymorphic loci; Ho = mean observed heterozygosity; He = mean expected heterozygosity.

Species	n	k	$P_{0.99}$	Ho	He
L. anus	8	1.1 (0.1)	14.3	0.024 (0.014)	0.023 (0.013)
L. platymetopon	16	1.2 (0.1)	14.3	0.039 (0.028)	0.050 (0.032)
Loricariichthys sp.	13	1.1 (0.1)	14.3	0.015 (0.009)	0.035 (0.023)

TABLE 4. Nei's unbiased genetic identity *I* is shown above the diagonal and genetic distance *D* is shown below the diagonal.

Species	*L. anus*	*L. platymetopon*	*Loricariichthys* sp.
L. anus	*****	0.329	0.663
L. platymetopon	1.112	*****	0.478
Loricariichthys sp.	0.411	0.738	*****

Various authors have demonstrated the value of data obtained through electrophoresis of enzymes for systematics and taxonomy of fish (Avise, 1974, 1994; Buth, 1984; Buth & Murphy, 1999; Ward & Grewe, 1995 and references therein). Besides the use of allozyme data as diagnostic characters, Buth & Murphy (1999) emphasize the importance of other electrophoretic characteristics such as tissue activity, heteropolymer formation, differences in ontogenetic expression and post-translational modifications.

According to Kettler *et al.* (1986) differences in the expression of a given enzyme in different tissues (differential tissue-specific expression, different tissular ontogenetic changes or heteropolymer fomation...) can be treated, as a set of characters for taxonomical studies, independently of the analysis of different enzymes in a given tissue (loci polymorphism, heterozygosity, presence/absence of diagnostic loci...).

The patterns of tissue- specific isozyme distribution is an indirect evidence of functional divergence (Basaglia, 1991a) and interspecific differences in these patterns can reveal informative evolutionary aspects of the gene-expression divergence in phylogenetic studies (Whitt, 1983; Basaglia, 1991b). For constructing a robust data set of tissue-expression one must get information about the activity of a given enzyme through the greatest possible number of tissues, and principally, in many species within a taxon. Since the data obtained here forms a part of a broader investigation on the family Loricariidae, containing many other species (Zawadzki, in preparation), the three tissues presented were found to be the most informative in regard to the number of loci, within the limits of time and money of the whole research.

Recent allozyme studies have been developed at population and species levels for neotropical fishes for example Fenerich-Verani *et al.* (1990a, 1990b); Almeida Val *et al.* (1990, 1992); D'Avila Ferreira *et al.* (1991); Monteiro *et al.* (1991); Renesto & Zawadzki (1997); Revaldaves *et al.* (1997); Almeida & Sodré (1998); Chiari & Sodré (1999); Lapenta *et al.* (1999); Zawadzki *et al.* (1999a, 1999b). Nevertheless, to the

present, no studies was conducted on the genus *Loricariichthys* preventing detailed comparison.

Here, two systems, MDH and LDH, present differences among the three species of *Loricariichthys* in relation to the patterns of tissue activity. Locus mMdh-A is expressed as an intense band in the liver of the three species and weak bands in the heart and muscle of *L. platymetopon*; while in the other two species its expression is limited to the liver. The LDH isozyme in liver tissue shows differences in relation to the heterotetramer interloci formation in these species of *Loricariichthys*, with a three banded pattern in *L. anus* and a two banded pattern in the other two species. In muscle tissue, all three species show a strongly expressed five banded pattern. Zawadzki *et al.* (in press) found in species of *Hypostomus* a single banded pattern in muscle and heart tissues corresponding to the homotetramer A_4 for *H.* aff. *commersonii* and *H. myersi* and to homotetramer B_4 for *H. derbyi*. Buth & Murphy (1999) state that multimeric and multiloci enzymatic systems can vary with regard to the production of interlocus heteropolymers and that this variation when established among the species can have systematic value. Murphy (1988) states that for fishes, there is a "general" trend in the LDH heteropolymer restriction, from the primitive five banded pattern to the advanced two banded A_4B_4 pattern condition. Thus, the LDH patterns observed for these species seem to corroborate the more basal position of Loricariinae relative to Hypostominae, or at a least to *Hypostomus* species, shown in recent phylogenetic analysis of the family (Schaefer, 1986, 1987; Montoya-Burgos *et al.*, 1997, 1998). Although, these enzymatic findings are not conclusive, it shows an open branch to one questioning the applicability of LDH isozymes in assaying Loricariidae phylogeny.

The presence of a single Esterase locus in these three species of *Loricariichthys* contrasts markedly with the six loci found by Lapenta *et al.* (1999) in *Hypostomus albopunctatus*. Duplication or silencing loci events in this enzymatic system can have occurred in the Loricariidae family and are also to be further investigated.

The heterozygosity found in the three species of *Loricariichthys* agree with the average 0.051, calculated by Ward *et al.* (1992) for freshwater fishes. The values are 0.023 for *L. anus*, 0.050 for *L. platymetopon*, and 0.035 for *Loricariichthys* sp. The greater values for the last two species could be explained by a more heterogeneous area sampled. However, Zawadzki *et al.* (1999b), studying sympatric loricariid catfish species of the genus *Hypostomus* in the Itaipu Hydroelectric Reservoir, found low heterozygosity for *H. albopunctatus* (0.015) and *H. microstomus* (0.016) and high heterozygosity for *H. regani* (0.103) and *H. ternetzi* (0.091), suggesting the predominance of historical factors rather than sampling artifacts in the determination of the genetic variability in some species of the family Loricariidae.

The unbiased genetic identity (I) (Nei, 1978), among the three species, is low. According to Thorpe (1982) and Thorpe & Solé-Cava (1994) 85% of *I* values between pairs of congeneric species exceed 0.35, and 76% of the values are above 0.4, while 77% of the values of *I* between different genera are below 0.35. Thus, the genetic identity between *L. platymetopon* and *L. anus* is as small as that between two distinct genera.

The allozyme discrimination among the three species of *Loricariichthys* was more efficient, in number of diagnostic characters, than the morphologic discrimination

of the same three species, as presented by Reis & Pereira (in press). Those authors found three morphological characters to discriminate between *L. platymetopon* and the new species, and two additional characters to separate the new species from the other two.

From these allozymatic diagnostic characters and from that obtained by Zawadzki *et al.* (1999a) which found allozyme genetic markers for three species of *Hypostomus* from the rio Iguaçu basin, we suggest the continuous use of allozyme electrophoresis as an auxiliary tool for the taxonomy of the family Loricariidae. It is worthwhile to point out that by enlarging the number of loci assayed as well as by screening and comparing several other loricariid species, the systematists could take profits from a still underexploited source of biological characters for taxonomic and phylogenetic analysis of this family.

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